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Metabolic inhibitors, elicitors, and precursors as tools for probing yield limitation in taxane production by Taxus chinensis cell cultures
AU Srinivasan, V.; Ciddi, V.; ***Bringi, V.*** ; Shuler, M. L.
CS School of Chemical Engineering, Cornell University, Ithaca, NY, 14853, USA
SO Biotechnol. Prog. (1996), 12(4), 457-465

Large scale production of secondary metabolites using plant cell cultures: Opportunities, realities and challenges.
AU Venkat, K.; ***Bringi, V.*** ; Kadkade, P.; Prince, C.
CS Phyton Inc., Itacha, NY 14850 USA
SO Abstracts of Papers American Chemical Society, (1997) Vol. 213, No. 1-3, pp. AGFD 54.
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I Large scale production of secondary metabolites using plant cell cultures: Opportunities, realities and challenges
AU Venkat, K.; ***Bringi, V.*** ; Kadkade, P.; Prince, C.
CS Phyton, Inc., Ithaca, NY, 14850, USA
SO Book of Abstracts, 213th ACS National Meeting, San Francisco, April 13-17 (1997), AGFD-054 Publisher: American Chemical Society, Washington, D. C.

Production of ***taxol*** by cell culture of Taxus. For development of techniques for industrial production
AU Hara, Yasuhiro; Yukimune, Yukihito
CS Mitsui Petrochem. Ind., Ltd., Yamaguchi, 740, Japan
SO Farumashia (1996), 32(7), 806-809
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TI Effect of picloram and methyl ***jasmonate*** on growth and ***taxane*** accumulation in callus culture of Taxus X media var. Hatfieldii.
AU Furmanowa, M.; Glowniak, K.; Syklowska-Baranek, K.
SO Plant cell, tissue and organ culture, 1997. Vol. 49, No. 1. p. 75-79
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AU Roberts, Susan C.; Shuler, Michael L.
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Large-scale plant cell culture

Susan C Roberts and Michael L Shuler*

Progress towards the commercial-scale use of plant cell cultures over the past three years has been significant. Elicitation, particularly with methyl jasmonate, has been effective at increasing the product yields of a wide variety of secondary metabolites, particularly when it is applied synergistically with enhancement strategies such as immobilization and *in situ* extraction. Rapid advances in understanding the regulation of the biosynthetic pathways of secondary metabolites are allowing the application of enhancement strategies to move from empirical to semirational. Much of this progress is exemplified by work on paclitaxel (Taxol), where yields have improved more than 100-fold in the past two years.

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Abbreviations

AS	anthranilate synthase
ppm	parts per million
TDC	tryptophan decarboxylase

Introduction

Plant cell culture technology shows promise for the large-scale production of valuable plant products; however, the commercial use of plant cell cultures is not routine because of difficulties in achieving acceptable, reproducible product levels in reasonable periods of time. Much work has been done in the three years since a prior review [1] to overcome this limitation and to determine conditions for commercial-scale reactors.

An increase in volumetric productivity is crucial for potential commercialization. Plant cell culture is a flexible system that is easily manipulated to increase product yields. This review focuses on strategies used to enhance secondary metabolite production that may contribute to the scale-up and commercialization of plant cell culture processes. Developments in the areas of bioreactor design, optimization of culture environment, elicitation and signal transduction, and biosynthetic pathway analysis are discussed. Paclitaxel (Taxol), a promising anticancer agent, is the focus of worldwide efforts in developing commercial-scale systems for its production. Recent progress on paclitaxel production is emphasized.

Bioreactor considerations

Several innovative reactor designs have been suggested that may be useful in the scale-up of shear-sensitive plant cell systems: a bubble-free loop fluidized bed bioreactor [2], an external loop air-lift bioreactor [3•], and a centrifugal impeller bioreactor [4•]. Additionally, organ cultures for the production of chemicals and the effects on reactor design have been considered by Doran [5]. Quantitative analysis of the effects of shear stress on plant cells has been discussed by Zhong *et al.* [6].

Taxus has been cultivated successfully for paclitaxel production in pneumatically mixed [7•], stirred tank [7•], and Wilson-type bioreactors [8]. The similarity of kinetics of paclitaxel production in all these reactors compared with shake flasks suggests that paclitaxel production is amenable to a variety of reactor configurations in bench-scale sizes and that for scale-up, shake flask data may be relevant. A continuous production system for paclitaxel production with a mesh-net cell separator was developed by Seki *et al.* [9] that increased productivity by a factor of ten compared with batch operation.

Unlike in *Taxus* cultures, when growth and ajmalicine production in *Catharanthus roseus* cultures were compared in shake flasks and bioreactors [10], growth was similar, but ajmalicine production was inhibited in the bioreactor. Ajmalicine production was restored with the recirculation of the ventilation gas. These results illustrate the potential importance of the gaseous phase in the scale-up of shake flask data to predict bioreactor performance.

Optimization of culture environment

In the case of ten Hoopen *et al.* [10], a change in gas phase composition was an unintentional consequence of change in reactor design. In some cases, the gas phase composition may be changed intentionally to achieve a desired change in cell metabolism. Gas phase composition has been shown to influence the timing and rate of paclitaxel production, with low oxygen concentrations promoting early production and high carbon dioxide concentrations inhibiting paclitaxel production [11•]. The most effective gas mixture was 10% v/v oxygen, 0.5% v/v carbon dioxide and 5 ppm (parts per million) ethylene.

Schlatmann *et al.* [12] addressed the question of what is the best time to start the production phase in a two-stage batch operation using *C. roseus* and ajmalicine production as a model system. The production phase was started with inocula of different ages and it was found that the amount of ajmalicine produced in the late stationary

phase inoculated cultures was greater than fourfold higher than the amount produced in the early stationary phase inoculated cultures. Nitrate concentration was found to be a key indicator of the start of the production phase. As soon as nitrate levels were depleted in the medium, secondary metabolism could be induced successfully. Whether or not nitrate might serve as a more general indicator of a switch to secondary metabolite production was not addressed.

The immobilization of plant cells often results in increased secondary metabolite production. One of the most commonly used immobilization techniques is calcium alginate entrapment. The role of calcium in immobilization-induced elicitation may be important. Gontier *et al.* [13] examined the effects of calcium and alginate separately and compared these effects to those of calcium alginate immobilization on the production of the alkaloids scopolamine and hoscyamine in *Datura innoxia* Mill. Calcium alone was found to be the most effective, inducing a tenfold increase in alkaloid production, probably because of the activation of N-methylputrescine transferase. Calcium was shown to be important for the successful elicitation of sesquiterpenes in *Hyoscyamus muticus* by fungal extracts of *Rhizoctonia solani* [14•]. Barium alginate immobilization did not induce an increase in sesquiterpene production.

The manipulation of the amount and source of sugar in cell cultures was studied as a factor for enhanced growth and secondary metabolite production [15–21]. Elevated sucrose levels were favorable in some cultures [15,20] and the addition of fructose promoted paclitaxel production in *Taxus* cell cultures [18,19].

Synergism of enhancement strategies

The combined effects of various enhancement strategies can stimulate secondary metabolite production many fold greater than any individual approach and this can be particularly valuable in large-scale systems. Recent examples of the successful use of this strategy include work by Choi *et al.* [22•] and Sajc *et al.* [23•]. The combined effects of immobilization in a spirally wound cotton cloth matrix, permeabilization with dimethyl sulfoxide (DMSO), and elicitation with *Verticillium dahliae* on gossypol production from *Gossypium arboreum* were compared in batch culture, an immobilized reactor with recycled batch operation, and an immobilized bioreactor with continuous operation [22•]. Elicitation had the maximum effect (eightfold induction) of all treatments and continuous operation was favorable over batch. When all of the treatments were combined, there was a 23-fold increase in gossypol production. Another recent example is the production of anthraquinones by *Frangula alnus* Mill. An external-loop air-lift bioreactor was utilized with calcium alginate immobilization and silicone oil *in situ* production extraction [23•]. Immobilization and silicone oil applied separately increased productivity up to fivefold, but when these strategies were combined, there was a 10–30-fold increase.

Elicitation and signal transduction

Elicitation is used to induce the expression of genes often associated with enzymes responsible for the synthesis of secondary metabolites. Gundlach *et al.* [24] demonstrated that jasmonic acid and its methyl ester are signal transducers in a wide range of plant cell cultures. These compounds accumulated rapidly and transiently when plant suspension cultures of *Rauvolfia canescens* and *Eschscholtzia californica* were treated with a yeast elicitor. Exogenously applied methyl jasmonate was shown to induce the production of secondary metabolites in 36 different plant species. In the past few years, jasmonic acid and methyl jasmonate have been shown to be inexpensive effective elicitors of secondary metabolite production in many other systems, including *Taxus*.

Paclitaxel production of 110 mg l^{-1} in two weeks could be induced from *Taxus media* with the addition of 100 μM methyl jasmonate [25••]. This rate is the highest productivity reported to date, although it is not the highest concentration of paclitaxel obtained, which was 153 mg l^{-1} [P1•]. Mirjalili and Linden [26•] have shown that exogenously applied methyl jasmonate at 10 μM results in a threefold increase in paclitaxel production from *Taxus cuspidata* cultures. When methyl jasmonate was combined with ethylene in an elicitation scheme, the result was a 19-fold increase in paclitaxel production. Both methyl jasmonate and ethylene are involved in the metabolic regulatory system in plants. This work points out the need to consider the interactions of multiple signals to the cell. Blechert *et al.* [27] showed that although natural jasmonic acid synthesis is initiated soon after fungal elicitation of *Agrostis tenuis* suspension cultures the synthesis rate is transient. This effect was shown to be highly specific, as jasmonic acid synthesis could not be reproduced with other types of stresses, including light, heavy metals, and cold or heat shock. Jasmonic acid was also shown to be an integral part of the signal transduction pathway leading to the induction of momilactone A biosynthesis [28•]. The treatment of elicited cells with ibuprofen, an inhibitor of jasmonic acid synthesis, reduced the content of endogenous jasmonic acid and momilactone A. This inhibition could be reversed with the addition of jasmonic acid. Also, 10 μM methyl jasmonate induced a fourfold increase in ajmalicine content and an increase in catharanthine concentration in cultures of *C. roseus* [15]. Methyl jasmonate is emerging as a useful tool to increase the production of secondary metabolites.

Elicitation has also been used extensively in the search for regulatory enzymes in the biosynthetic pathways of secondary metabolites; however, control of the elicitation processes has yet to be optimized. Archambault *et al.* [29] measured a twofold increase in sanguinarine production in cultures of *Papaver somniferum* with chitin elicitation and discovered that the most successful elicitation schemes involved the addition of elicitor before phosphate deple-

tion. Cell cultures of carrot were elicited for anthocyanin production [30] with culture filtrates and cell extracts of bacteria and yeast as well as various abiotic ions. Calcium was the most effective, promoting a twofold increase in anthocyanin production, indicating that it may play a more general role as a signal molecule in the elicitation process, although the interpretation of these results may be complicated by the heterogeneous mix of signal molecules in natural culture filtrates. *Taxus* suspension cultures were elicited with cell extracts and culture filtrates of four different fungi and arachidonic acid [31•]. Three categories of elicitors were identified: those eliciting only paclitaxel, those eliciting only other taxanes, and those eliciting both paclitaxel and other taxanes equally, indicating different biosynthetic sites of action. Interestingly, both arachidonic acid in the above study [31•] and the addition of methyl jasmonate in the study of Yukimune *et al.* [25••] resulted in preferential increases in paclitaxel over other taxanes.

A method for predicting elicitor dosages for plant cell culture reactor systems was developed by Singh *et al.* [32]. A physical interpretation of elicitation is presented as an elicitor–receptor binding event. Elicitor dosage was found to be dependent on both the tissue density and the free elicitor concentration in the medium. By mathematically characterizing elicitation, the amount of elicitor to be added to cell cultures can be easily determined with reduced experimentation time.

Biosynthetic pathway analysis and control

A lot of recent research on plant cell cultures has focused on determining the control of production of secondary metabolites by identifying the rate-limiting steps in the biosynthetic pathways. Several approaches can be effective: elicitation followed by monitoring the activities of pathway enzymes, measuring enzyme levels in cell lines of different biosynthetic capabilities, addition of precursors, and the transformation and overexpression of pathway genes. This trend of investigating the control of secondary metabolite production is important in moving plant cell technology from a soley empirical approach to metabolite production towards a semirational approach. Hashimoto and Yamada [33] have provided an excellent overview on the molecular aspects of alkaloid biosynthesis that includes a discussion of their pioneering work on the production of tropane alkaloids in genetically engineered root cultures.

Bohlmann and Eilert [34] have investigated the branch point of the shikimate pathway for control of the production of acridone epoxides, furoquinoline and furanocoumarins by *Ruta graveolens* L. They determined, after fungal elicitation, that chorismate mutase is not a key enzyme in the induction of furanocoumarins but anthranilate synthase (AS) does play a key role in regulating the production of acridone epoxides. Currently, the purification of AS and cDNA cloning are underway to better understand the regulatory role of AS. This study provides a good model for an approach to identify rate-limiting steps.

Dagnino *et al.* [35•] studied two cell lines of *Tabernaemontana divaricata* with different biosynthetic capabilities for the production of terpenoid indole alkaloids. By adding the precursor loganin, pathway enzyme levels were not increased, but alkaloid accumulation in both cell lines was raised to similar levels (fivefold increase for the high-accumulating cell line and 100-fold increase for the low-accumulating cell line), indicating that biosynthesis is limited by the availability of precursors.

The enzyme tryptophan decarboxylase (TDC) catalyzes a key step in the biosynthesis of terpenoid indole alkaloids in *C. roseus* by converting tryptophan into tryptamine. This enzyme is present at low levels and may be a limiting factor in alkaloid biosynthesis. Goddijn *et al.* [36•] increased TDC levels by transformation and overexpression, which led to an increase in tryptamine content but no increase in alkaloid accumulation, indicating that the TDC-catalyzed reaction is not rate limiting for the production of these alkaloids, but is rate controlling for a precursor compound.

Additional insights into the metabolic control of terpenoid production in *C. roseus* cell cultures are found in the work of Moreno *et al.* [37•]. There was no increase in ajmalicine accumulation upon elicitation with a cell-free filtrate of *Pythium aphanidermatum*. Some enzymes early in the indole alkaloid pathway were induced upon elicitation and others were inhibited whereas enzymes further along were not elicited. These results indicate that there is a limitation in the terpenoid pathway that may cause a shortage of secoiridoid precursors for alkaloid biosynthesis. It also demonstrates the limitations of the use of nonspecific elicitors because levels of desired enzymes were suppressed in some cases and enhanced in others.

If a secondary product contains an element in its molecule that is derived from an environmental mineral nutrient, control mechanisms can be studied by varying the mineral concentration. Thiophene contains sulphur and its biosynthesis in *Tagetes* can be controlled by sulfate concentration in the medium [38]. Reduced sulfate concentration in the medium decreased thiophene accumulation fourfold.

Characterization of the paclitaxel biosynthetic pathway is a critical factor in attempts to increase yields. Elicitation, inhibition of metabolic steps, and precursor feeding were used to better understand the paclitaxel biosynthetic pathway [39••]. Paclitaxel production and isopentenyl pyrophosphate source were both found to be primarily plastidic. The addition of various inhibitors and a comparison of kinetic profiles suggest that baccatin III need not be a direct precursor of paclitaxel. Conceptual models were formulated to describe carbon flow and simple

mathematical simulations were performed. Model results and experimentation suggest that paclitaxel production is limited by the ability of the cells to convert phenylalanine to phenylisoserine. This methodology can be useful in predicting rate-limiting steps, particularly when details of pathways and compartmentation are unknown.

The enzymes catalyzing the initial steps of paclitaxel biosynthesis have been identified [40–42]. Taxadiene synthase catalyzes the initial conversion of geranylgeranyl pyrophosphate, the universal precursor of diterpenoid compounds, to 2-taxa-4(5),11(12)-diene. The next step is the hydroxylation of taxa-4(5),11(12)-diene to taxa-4(20),11(12)-dien-5a-ol by a cytochrome P450 enzyme (taxadiene-5-hydroxylase). It is speculated that additional oxygenation steps may be catalyzed by similar P450 moieties. These initial two steps were found to be very slow compared to subsequent metabolic transformations. Recently, the cDNA encoding taxadiene synthase has been isolated [43] and the authors speculate that it should soon be possible to engineer cells that overexpress these two enzymes, resulting in enhanced paclitaxel productivity.

A very important insight into paclitaxel synthesis comes from radiolabeled studies with *Taxus chinensis* in which it was shown, contrary to expectations, that the taxane ring system is not synthesized through the mevalonate pathway [44•]. This is the first report of an alternative pathway being used in plants and may be important for other plant products.

Conclusions

The use of plant cell culture for the production of chemicals and pharmaceuticals has made great strides building on advances in plant sciences. Better molecular understanding of elicitation and signal transduction in plants is emerging. The use of methyl jasmonate is one strategy that appears to be broadly useful and can result in rapid improvements in productivity in a short period of time. The interrelationship of methyl jasmonate signaling with other signal compounds is beginning to emerge and will supplement the widely demonstrated strategy of using multiple productivity enhancement techniques to achieve synergistic increases in production.

Further, the increased use of genetic tools and an emerging picture of the structure and regulation of pathways for secondary metabolism will provide the basis to move from a brute-force empirical approach for the optimization of production conditions to a semirational one; one can then predict a great reduction in the time required to move from the establishment of a culture to the point when conditions are optimal for the production of commercially acceptable levels of product.

Although further improvements in bioreactor design may be anticipated, these studies will be important primarily as vehicles that allow the more effective application of synergistic product-enhancement strategies. Particularly important will probably be the need to better control gas phase composition, to facilitate application and removal of elicitors, and, in some cases, to facilitate the use of *in situ* product-removal strategies.

The increased appeal of natural products for medicinal purposes coupled with the low product yields and supply concerns of plant harvestation has renewed interest in large-scale plant cell culture technology. The anticancer agent paclitaxel has been the focus of recent research, which has shifted from establishing paclitaxel-producing cultures to enhancing productivity in these cultures.

Three years ago, the presence of even low levels of paclitaxel was considered significant and two years ago the majority of reports described low paclitaxel levels [19,45,46]. Now, reports of paclitaxel levels of 10–22 mg l^{-1} are common from academic laboratories [8,18,21,26•] and much higher levels (153 mg l^{-1} [P1•] and 110 mg l^{-1} [25••]) have been reported from industrial groups. Bristol-Meyers Squibb (Princeton, NJ, USA) announced in 1995 that they were licensing the Phyton Inc (Ithaca, NY, USA) plant cell culture process for paclitaxel production. The past three years have witnessed tremendous progress towards the commercialization of plant cell culture for the production of a pharmaceutical. Should the plant cell culture process for paclitaxel become a successful reality, it will open doors for the serious consideration of plant cell culture for the commercial-scale production of other pharmaceuticals. Should the process for paclitaxel falter, it will significantly reduce interest in large-scale plant cell culture. Progress in the next three years will be critical.

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Patents

- • of special interest
- •• of outstanding interest

P1. • Bringi V, Kadkade PG, Prince CL, Schubmehl BF, Kane EJ, Roach B: **Enhanced production of Taxol and taxanes by cell cultures of *Taxus* species.** 1995, US5407816.
This patent reports the highest level of paclitaxel production (153 mg l^{-1}) in the literature. It examines a wide variety of enhancement strategies.